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(11) Publication number:

0 186 084
A2

(12)

EUROPEAN PATENT APPLICATION

(21) Application number: 85116002.8

(51) Int. Cl.⁴: A 61 K 35/30
C 12 N 5/00

(22) Date of filing: 16.12.85

(30) Priority: 24.12.84 US 685923

(43) Date of publication of application:
02.07.86 Bulletin 86/27

(84) Designated Contracting States:
CH DE FR GB IT LI NL

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(54) Brain-derived growth factor.

(57) Brain-derived acidic fibroblast growth factor, (aFGF), is an active mitogen for vascular endothelial cells in culture and is useful for growth of such cultures for coverage of polymeric vascular grafts; growth of such cultures on tubular supports for production of blood vessels for implantation.

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TITLE OF THE INVENTIONBRAIN-DERIVED GROWTH FACTORSUMMARY OF THE INVENTION

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Brain-derived acidic fibroblast growth
factor, (aFGF), is an active mitogen for vascular
10 endothelial cells in culture and is useful for growth
of such cultures for coverage of polymeric vascular
grafts; growth of such cultures on tubular supports
for production of blood vessels for implantation; and
stimulation or facilitation of blood vessel growth
15 and repair in vivo.

BACKGROUND OF THE INVENTION

The brain-derived aFGF purification and
wound healing activity of this invention was
20 described in U.S. Patent 4,444,760, issued April 24,
1984 to Kenneth A. Thomas, Jr. The protein is also
described by Thomas et al., Proc. Natl. Acad. Sci.,
USA 81, 357-361 (1984). The same or similar proteins

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may also be present in other partially purified extracts from the central nervous system and other organs. See, for example, Maciag et al., Science, 225, 932 (1984).

5 Although the growth of vascular endothelial cells was accomplished in the past using very high concentrations of fetal calf or adult bovine serum (10-30%) the results were variable, depending on the particular lot of calf serum, and the rate of cell
10 growth was generally slow. Now, with the present growth factor rapid growth rates are achieved with serum levels from 0 to 2%.

 This novel method of reproducible stimulation of vascular endothelial growth, mediated
15 by pure protein mitogens permits:

1) Covering synthetic polymeric vessels with non-thrombogenic vascular endothelial cells from the host animal, including human, whereby many or all of the clotting problems associated with synthetic
20 vessel grafts are obviated;

2) production of vessels in vitro by growth of host vascular endothelial cells on tubular supports, for implantation back into the same host animal, including human, whereby immunological rejection of
25 the implant will be obviated and the frequent limited supply of good vessels within the patient for transplant will also be obviated; and

3) stimulation or facilitation of blood vessel growth and repair in vivo, whereby the flow of blood
30 to tissues deprived of adequate oxygen and/or other blood borne components is increased.

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DETAILED DESCRIPTION OF THE INVENTION

The brain-derived aFGF useful in the novel methods of this invention is prepared as described in U.S. Patent 4,444,760 the disclosure of which is
5 incorporated herein by reference.

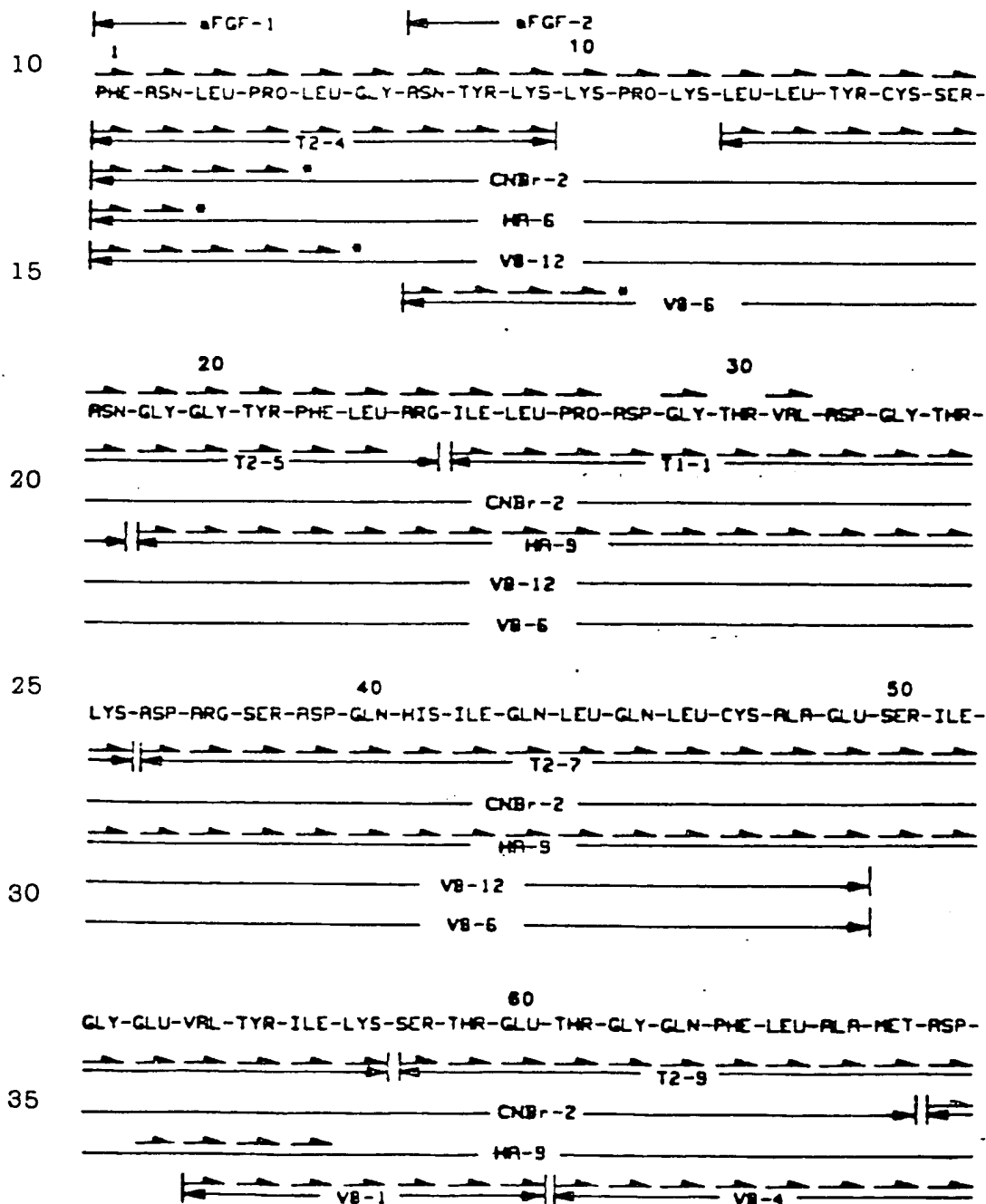
The complete amino acid sequence has been determined for aFGF. Sequence determinations of reduced and carboxymethylated protein have revealed two amino termini. The longer sequence, aFGF-1,
10 contains six amino terminal residues not found on the shorter aFGF-2 form. The relative amounts of these two microheterogeneous forms of the mitogen vary from one purification to another but are closely correlated in amount to the abundance of the two
15 bands of protein previously seen by electrophoresis in SDS polyacrylamide gels (Thomas, et al., Proc. Natl. Acad. Sci. USA, 81, 357 (1984)). As expected, the amount of the longer amino terminal sequence correlates with the relative quantity of the higher
20 mass band on the SDS gels. If the length of the polypeptide chain at the amino termini is the only difference between the two microheterogeneous forms observed on the SDS gels, then the mass difference between them is 642 daltons, rather than the
25 previously estimated 200 daltons based on SDS gel migration distances. It is assumed that the amino terminal heterogeneity is the result of limited proteolysis either in vivo or during purification.

The complete amino acid sequence was
30 determined from sequences of the amino termini and overlapping peptides generated by proteolytic cleavages with trypsin (T), Staphylococcus aureus V8 Protease (V8), hydroxylamine (HA) and cyanogen-bromide

- 1 (CN). The carboxyl terminal sequence of the whole protein was confirmed by timed carboxypeptidase A digestion.

- 5 In a search of the current Dayhoff protein data bank, aFGF is unique compared to the approximately 2000 protein sequences contained in that list.

The sequence is as follows:



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1 THR-ASP-GLU LEU-LEU-TYR-GLY-SER-GLN-THR-PRO-ASN-GLU-GLU-CYS-LEU-PHE-
70 80
T2-9
CNBr-1
HA-9
5 V8-4 V8-3
90 100
LEU-GLU-ARG-LEU-GLU-GLU-ASN-HIS-TYR-ASN-THR-TYR-ILE-SER-LYS-LYS-HIS-
T1-2
CNBr-1
HA-9
10 V8-2
110
ALA-GLU-LYS-HIS-TRP-PHE-VAL-GLY-LEU-LYS-LYS-ASN-GLY-ARG-SER-LYS-LEU-
T2-7
T2-1
CNBr-1
HA-9
15 HA-7 V8-8
120 130
GLY-PRO-ARG-THR-HIS-PHE-GLY-GLN-LYS-ALA-ILE-LEU-PHE-LEU-PRO-LEU-PRO-
T2-11
CNBr-1
HA-7
20 V8-8
140
VAL-SER-SER-ASP
T2-11
CNBr-1
HA-7
25 V8-8
30

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1 Peptide sequences that were prematurely
terminated because they were recognized to begin at
one of the two previously determined amino termini are
marked with asterisks following the last degradation
5 cycle.

The novel method for the stimulation of
vascular endothelial cells comprises treating a
sample of the desired vascular endothelial cells in a
nutrient medium with aFGF at a concentration of about
10 1-10 ng/ml.

If the vascular endothelial cell growth is
conducted in vitro, the process requires the presence
of a nutrient medium such as Dulbecco's modified
Eagle's medium or modification thereof and a low
15 concentration of calf or bovine serum such as about 0
to 2% by volume. Preservatives such as a penicillin-
streptomycin combination or other broad spectrum
antibacterials are also employed. It is preferred to
have about 10 to 100 ug/ml of heparin present also.

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The novel method of this invention is useful for the coverage of artificial blood vessels with endothelial cells. Vascular endothelial cells from the patient would be obtained by removal of a small segment of peripheral blood vessel or capillary-
5 containing tissue and the desired cells would be grown in culture in the presence of aFGF and any other supplemental components that might be required such as heparin and/or serum. After growth of
10 adequate numbers of endothelial cells in culture to cover the synthetic polymeric blood vessel the cells would be plated on the inside surface of the vessel. Prior coating of the artificial vessel either covalently or noncovalently, with either heparin or
15 proteins such as fibrin, collagen, fibronectin or laminin would be performed to enhance attachment of the cells to the artificial vascular surface. The cell-lined artificial vessel would then be surgically implanted into the patient and, being lined with the
20 patients own cells, would be immunologically compatible. The non-thrombogenic endothelial cell lining should decrease the incidence of clot formation on the surface of the artificial vessel and thereby decrease the tendency of vessel blockage or
25 embolism elsewhere.

The novel method is also useful for the production of artificial vessels. Vascular endothelial cells and smooth muscle cells from the patient would be obtained and grown separately in
30 culture. The endothelial cells would be grown in the presence of the aFGF as outlined above. The smooth muscle cells would be grown in culture by standard techniques. A tubular mesh matrix of a biocompatible

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polymer (either a synthetic polymer, with or without a coating of either heparin or specific attachment proteins, or a non-immunogenic biopolymeric material such as surgical suture thread) would be used to support the culture growth of the smooth muscle cells on the exterior side and vascular endothelial cells on the interior surface. Once the endothelial cells form a confluent monolayer on the inside surface and multiple layers of smooth muscle cells cover the outside, the vessel is implanted into the patient.

The novel method can also be used for the induction of vascular growth. The pure growth factor or the equivalent human protein would be used to induce and promote the growth of blood vessels in the patient. The mitogen would be administered along with any necessary stabilizers and enhancers of activity including heparin, at the site of desired vascular growth. For applications involving neovascularization of surface wounds, such as abrasions or burns, the formulation would be applied directly in a slow release polymer at a rate of about 1-100 ng/day/cm² of injured surface. For internal vascular growth, the formulation would be released directly into the region to be neovascularized either from implanted slow release polymeric material or from slow release pumps. The release rate in either case is preferably about 100 ng - 10 ug/day/cm³ of injured tissue.

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EXAMPLE 1MITOGENIC RESPONSE OF FETAL BOVINE THORACIC
AORTIC ENDOTHELIAL CELLS TO aFGF

5 Fetal bovine thoracic aortic endothelial
cells (AG4762, N.I.A. Aging Cell Repository,
Institute for Medical Research, Camden, New Jersey)
were assayed after 38 cumulative population doublings
10 in vitro. The cells were plated in 6-well Costar
plates at 2×10^3 cells/cm² in 20% heat
inactivated fetal calf serum in Dulbecco's modified
Eagle's medium (DMEM, Gibco) and changed to 1% serum
18 hours later. All media were supplemented with
glutamine and penicillin-streptomycin as previously
15 described. Either pure aFGF diluted in 100 ul of 1
mg bovine serum albumin (Sigma) per ml of DMEM or
serum samples were added to each well along with 1.6
uCi of ³H-thymidine (New England Nuclear) and 45 ug
of unlabeled thymidine in 40 ul of DMEM. After a 48
20 hour incorporation period, the cells were washed,
lysed and 75% of the trichloroacetic acid
(TCA)-insoluble DNA from pure growth factor (-●-) or
serum (-■-)-stimulated cells was counted.

The increase in endothelial cell population
25 at various concentrations of aFGF was measured by
measuring the uptake of tritiated thymidine. The
results are shown in Figure 1, below.

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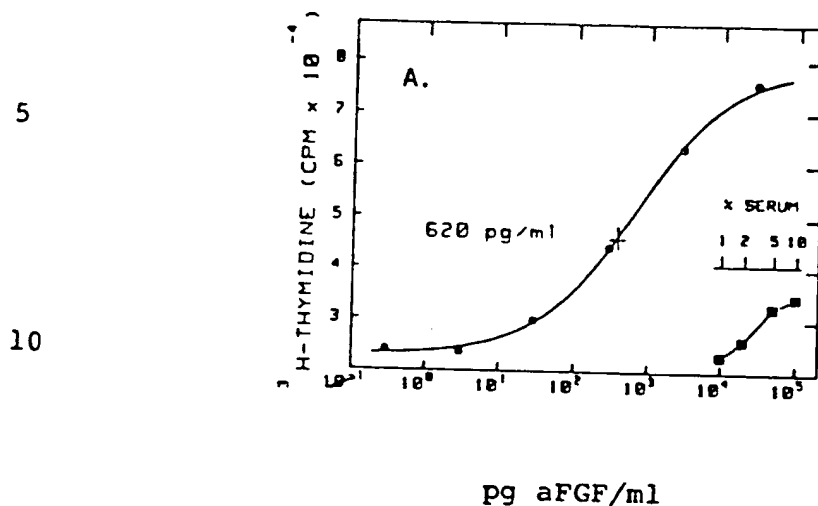


Figure 1

EXAMPLE 2MITOGENIC RESPONSE OF MOUSE LUNG CAPILLARY
ENDOTHELIAL CELLS TO aFGF

20 Mouse lung capillary endothelial cells were
plated at 2.6×10^4 cells/cm² in 0.5 ml/well in
24-well Costar dishes and grown to confluence in 10%
charcoal-treated calf serum (HyClone Laboratories,
Logan, Utah) in DMEM, lowered to 0.5% serum after 72
25 hours and allowed to become quiescent over 48 hours.
Either serum or the pure aFGF were added in 50 μ l as
described above followed 18 hours later by a 4 hour
pulse of ^3H -thymidine (20 μ l of 100 μ Ci/ml
 ^3H -thymidine in Gibco phosphate buffered saline).
30 The cells were processed and radioactivity counted as
described in Example 1, and the results were as shown
in Figure 2.

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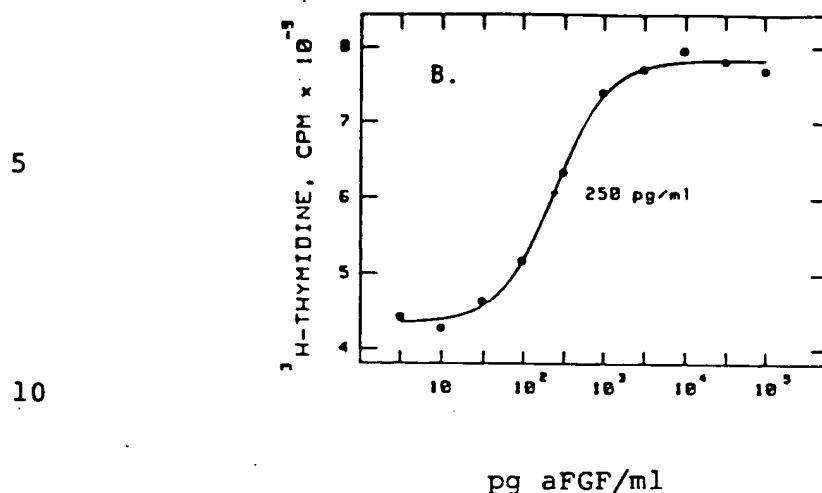


Figure 2

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EXAMPLE 3

Angiogenic Activity of aFGF

Chicken Egg Angiogenesis Bioassay

During the sustained vascular growth,

20 endothelial cells are observed to actively proliferate. Therefore, we tested the ability of the purified mitogen to induce blood vessel growth in the chicken egg chorioallantoic membrane angiogenesis assay. Based on previous reports that crude tumor

25 angiogenesis factor was significantly more active with coadministered heparin, we tested the vascularization response of heparin alone and heparin plus pure aFGF.

30 Three-day old chicken embryos were removed from their shells and grown in Handiwrap pouches suspended inside paper cups. The tops of the cups were covered with Handiwrap, and the eggs were incubated at 37°C in a tissue culture incubator for

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5-6 days. Either 1 μ g of pure aFGF in about 30 μ l of the HPLC elution solvent (7 mM trifluoroacetic acid/33% acetonitrile) or an identical HPLC solvent control solution were mixed with an equal volume of
5 2% low-gelling temperature agarose (Miles) dissolved in lactated Ringer's solution (Abbott) containing 10 μ g of heparin (from porcine intestinal mucosa; Sigma grade 1). Droplets (60 μ l) were allowed to gel on the center of sterile plastic 1.3-cm diameter Thermanox
10 tissue culture coverslips (Miles), and at least part of the volatile acetonitrile evaporated by aeration for 15-30 minutes under a plenum of sterile air in a tissue culture hood. The coverslips were positioned, pellet down, over the chorioallantoic membrane of the
15 eggs and incubated for 3 days. Eggs containing large white focal regions under the coverslips at the end of the assay, presumably formed by inflammatory cells, were discarded. The chorioallantoic membranes were examined microscopically and scored for the
20 proliferation of fine capillaries under the center of cover-slips by observers who did not know the contents of the agarose pellets.

A 10 μ g dose of heparin per egg was inactive but the same amount of heparin plus 1 μ g of aFGF per
25 egg appeared to enhance the growth of small capillaries at the site of application with no sign of inflammation (Table 1). The assay is reproducible, the results being a composite of three separate assays with different samples of aFGF.
30 Control and positive angiogenic responses show the extent of capillary proliferation induced by aFGF. The mitogen is, therefore, a potent angiogenic protein in the presence of heparin.

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TABLE 1

Angiogenic Activity of aFGF

	<u>Sample contents</u>	<u>Angiogenic response</u>	
		<u>Negative</u>	<u>Positive</u>
5	Control	15	0
	aFGF	2	10

These data are a composite of three separate experiments. Using t-distribution statistics, the group of mitogen-stimulated eggs was calculated to be different from the control population with a confidence level of 99.9%.

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WHAT IS CLAIMED IS:

1. A novel method for the stimulation of growth of vascular endothelial cells which comprises
5 treating a sample of the desired endothelial cells in a nutrient medium with aFGF at a concentration of about 1-10 ng/ml.

2. A method for the stimulation of the
10 growth of vascular endothelial cells in vitro which comprises treating a sample of vascular endothelial cells in a nutrient medium, comprising 2% or less of bovine serum, glutamine and a broad spectrum
antibiotic with aFGF at a concentration of about 1-10
15 ng/ml.

3. The method of Claim 2 wherein cells from a vascular explant are grown on the surface of a synthetic polymeric vessel for implanting in the host.
20

4. The method of Claim 2 wherein endothelial cells from a vascular explant are grown on the interior surface of a biocompatible tubular mesh support and smooth muscle cells are grown on the
25 external surface of the tubular mesh support for implanting in the host.

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